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Chloroplast immunity illuminated

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Summary

The chloroplast has recently emerged as pivotal to co-ordinating plant defence responses and as a target of plant pathogens. Beyond its central position in oxygenic photosynthesis and primary metabolism – key targets in the complex virulence strategies of diverse pathogens – the chloroplast integrates, decodes and responds to environmental signals. The capacity of chloroplasts to synthesize phytohormones and a diverse range of secondary metabolites, combined with retrograde and reactive oxygen signalling, provides exquisite flexibility to both perceive and respond to biotic stresses. These processes also represent a plethora of opportunities for pathogens to evolve strategies to directly or indirectly target 'chloroplast immunity'. This review covers the contribution of the chloroplast to pathogen associated molecular pattern and effector triggered immunity as well as systemic acquired immunity. We address phytohormone modulation of immunity and surmise how chloroplast-derived reactive oxygen species underpin chloroplast immunity through indirect evidence inferred from genetic modification of core chloroplast components and direct pathogen targeting of the chloroplast. We assess the impact of transcriptional reprogramming of nuclear-encoded chloroplast genes during disease and defence and look at future research challenges.

II. Introduction

A plant's initial response to a broad spectrum of different stresses, including pathogens, is through integrated signalling modules that recognize a common set of second messengers (calcium, reactive oxygen species (ROS), nitric oxide (NO) and lipid molecules), often incorporating kinase-based signal transduction cascades. Understanding how cells specify the timing, amplitude and duration of signal outputs, and decode and integrate these signals locally and distally remains a key challenge in plant biology. What is

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often neglected is that these signals are perceived not only at the cell surface and/or in the nucleus, but also by other organelles, which collectively contribute to orchestrating an effective response.

1. Plant immunity, more than membrane to nuclear signalling

Put simply, plant immunity comprises three core modules. Predominately membrane-localized pattern recognition receptors (PRRs) perceive pathogen-associated molecular patterns (PAMPs) activating PAMP-triggered immunity (PTI). Pathogens deliver effectors (generally proteinaceous but also small molecules) directly or indirectly into the cell to collectively suppress PTI, often targeting the PRRs and their coreceptors. This effector triggered suppression (ETS) can be successfully overcome by intracellular plant disease resistance (R) proteins which activate effector-triggered immunity (ETI) effectively containing and eliminating the invading pathogen through a programmed cell death process known as the hypersensitive response (HR)(Jones & Dangl, 2006). However, there is a growing acceptance that PTI and ETI are not two distinct processes but are somewhat interdependent and contribute as a continuum to host immune responses (van der Burgh & Joosten, 2019). Superimposed on ETI is the initiation and establishment of broad-spectrum systemic immunity known as systemic acquired resistance (SAR) (Shine *et al.*, 2019). Because of the localization of PRRs and signalling components of ETI, most of the innate immunity research has been focused on the cell membrane, the nucleus and the role of MAPK (mitogen-activated protein kinase) signalling cascades in unravelling plant immunity mechanisms.

2. The chloroplast is a key hub in coordinating effective plant immune responses

Aside from oxygenic photosynthesis, chloroplasts act as both environmental signal integrators and metabolic hubs. Chloroplasts not only link to primary metabolism but synthesize phytohormones, fatty acids, amino acids and a plethora of other secondary metabolites. This therefore provides unprecedented flexibility in fine tuning complex signalling to specific environmental stresses, and the capacity to rapidly modulate and redeploy metabolic signalling. This review will focus on the role of the chloroplast in disease and defence and seek to provide the reader with an overview of current knowledge of chloroplast immunity. We will examine evidence of a pivotal role for chloroplasts both in orchestrating an effective immune response and as a pathogen target, to suppress immunity. Pathogens probably also reconfigure primary metabolism for nutrition, although experimental insight into this is limited. We will additionally touch on current concepts in retrograde signalling and draw parallels with other stress process that impact chloroplast homeostasis to explore commonalities in signalling responses.

3. Chloroplasts in plant immunity: an historical overview

In the past decade, the chloroplast has emerged as a central player in plant defence, initially in the context of its identification as a genuine effector target but more recently in recognition of its contribution to defence. The importance of the chloroplast in immunity has been known for a long time. Kupeevicz (1947) first reported that viruses and other plant pathogens alter chlorophyll (Chl) accumulation during infection. By the 1990s, viral proteins, such as the coat protein of Tobacco Mosaic Virus (TMV), were identified within the chloroplast (Banerjee & Zaitlin, 1992). In comparison to virus research (which we only use here as exemplars), studies on how bacteria, fungal and oomycete pathogens target the chloroplast were limited until the emergence of 'effector biology' in the early 2000s.

4. The complexity of chloroplast immunity: where to begin?

We aim to leave readers with two key messages, the first being that chloroplast-derived reactive oxygen species (cROS) play a pivotal role in establishing effective plant immunity and, secondly, that pathogen effectors directly and indirectly target chloroplast processes to suppress immunity. Obviously, to effect these changes a plethora of processes are activated or suppressed. While recognising this is still an embryonic field, we will draw on relevant examples to provide insight into current state-of-knowledge of the complexity of chloroplast processes modified and known components targeted. We first briefly overview phytohormone modulation of immunity, and the contribution of the chloroplast to PTI, ETI and SAR in the context of cROS, and how effectors modulate/facilitate this.

We next document potential processes contributing to chloroplast immunity that have been revealed using genetic approaches, with a strong focus on the role of cROS. We then provide a comprehensive overview of proteinaceous pathogen effectors targeted to the chloroplast and their targets, if known. Finally, we examine transcriptional control of nuclear-encoded chloroplast genes in PTI and ETI and touch on the emerging role of subcellular reorganization. Chloroplast retrograde signalling has recently been comprehensively reviewed (Chan *et al.*, 2016; de Souza *et al.*, 2017), including possible roles for metabolites in immune signalling (Fernandez & Burch-Smith, 2019) and hence this is not addressed here, other than to highlight specific examples.

We have tried to illustrate a variety of key immune processes that impact the chloroplast throughout the review. A powerful technique to visualize the impact of pathogens on chloroplast physiology is through F_v/F_m measured by Chl fluorescence imaging (Baker, 2008). F_v/F_m provides sensitive quantitative temporal–spatial measurements of changes in the maximum (dark-adapted) quantum efficiency of photosystem II (PSII; a sensitive indicator of damage/downregulation of photosynthesis), while simultaneously enabling imaging of pathogen challenges in real time and, thus, is increasingly being used to monitor pathogen infection dynamics (de Torres Zabala *et al.*, 2015). Fig. 1 illustrates suppression of PTI by the virulent phytobacterial pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst*) both visually (Fig. 1a) and quantitatively (Fig. 1b) and its relationship to *in planta* bacterial multiplication (Fig. 1c). F_v/F_m can also effectively capture changes in chloroplast physiology caused by fungal challenges (Fig. 1d).

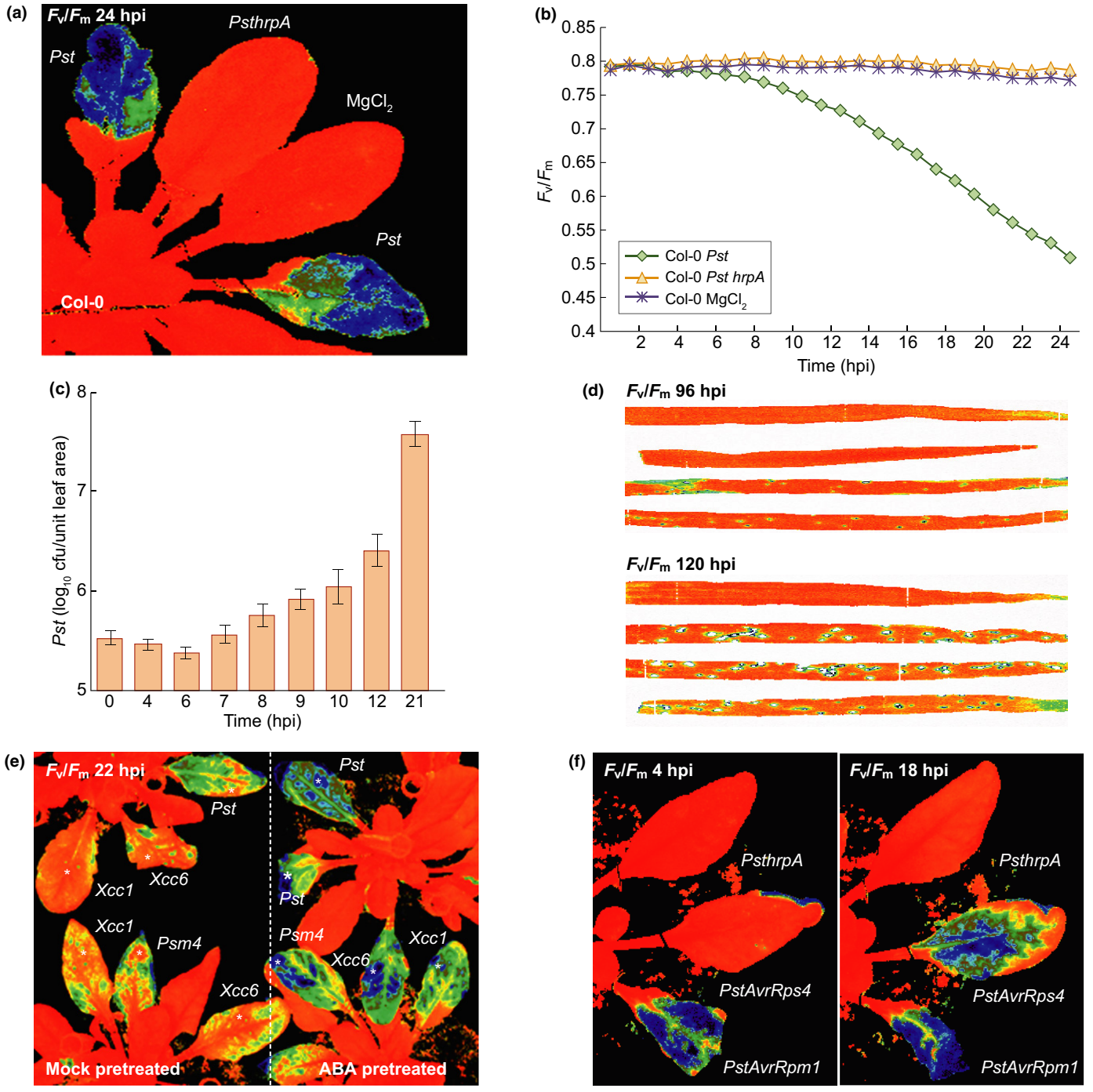

Fig. 1 Photosystem II quantum efficiency (F_v/F_m) captures early chloroplast changes in response to virulent and avirulent pathogens. (a,b) Challenge with the virulent apoplastic bacterial phytopathogen *P. syringae* pv. *tomato* DC3000 (*Pst*) but not mock ($MgCl_2$) or the disarmed *hrpA* mutant results in reduced F_v/F_m 7–8 hpi (h post-infection) as illustrated visually (a) or quantitatively during disease establishment (b). (c) *Pst* multiplication significantly increase above initial inoculation levels at 8 h post-infiltration, coincident with reduction in F_v/F_m. Error bars, ± SD. (d) Spray infection with spores of the virulent rice pathogen *Magnaporthe oryzae* Guy11 similarly induces localized decreases in F_v/F_m. (e) Challenge with the vascular pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) or *P. syringae* pv. *maculicola* suppresses F_v/F_m during infection, the extent of which is directly correlated with virulence of the strains. Pretreatment with ABA, which is rapidly induced *de novo* following virulent bacterial infections, dramatically enhances suppression of F_v/F_m in both *Xcc* and *Pst*. (f) ETI induced either by RPM1 or RPS4 following challenge with *Pst* carrying the respective avirulence genes, *AvrRpm1* or *AvrRps4*, causes a rapid suppression of F_v/F_m, the timing of which is unique to the specific R protein and correlates with speed of HR development. Kindly provided by: (a–c, f) M. Grant & S. Breen; (d) G. Littlejohn; (e) de Torres *et al*. (2015: Fig. S4).

III. Hormones and chloroplasts, a well-established link in plant immunity

Being the main site of phytohormone precursor synthesis, the chloroplast is central to integrating signals from PTI and ETI and an obvious target for effector modulation. Hormonal crosstalk in plant–microbe interactions is now well established (Robert-Seilaniantz *et al.*, 2011; Burger & Chory, 2019). Thus, here we only briefly overview the core roles of, or selected new insights into, the three key immunity modulating hormones salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) to provide context for further reference in later sections.

1. Salicylic acid

Salicylic acid (SA) is the archetypal defence hormone effective against biotrophic and hemibiotrophic pathogens (Ding & Ding, 2020). Rapid SA biosynthesis in response to pathogens occurs through formation of isochorismate by the chloroplast-localized isochorismate synthase (ICS). The decades long challenge to understand how isochorismate was converted to SA was recently resolved. EDS5 (ENHANCED DISEASE SUSCEPTIBILITY 5) exports isochorismate to the cytosol where the amidotransferase PBS3 (avrPphB SUSCEPTIBLE3), originally identified in a genetic screen for loss of RPS5-specified resistance (Warren *et al.*, 1999), catalyses its conjugation to glutamate, forming isochorismate-9-glutamate, which spontaneously decomposes into SA (Rekhter *et al.*, 2019; Torrens-Spence *et al.*, 2019). SA may directly interfere with pathogen virulence strategies through its interaction with nonexpressor of pathogenesis-related gene (NPR) SA receptors. Increases in SA inhibit the transcriptional corepressors NPR3 and NPR4, but activate the transcriptional coactivator NPR1, collectively inducing SA responsive defence genes, including key regulators of plant immunity (Ding *et al.*, 2018). SA can also function indirectly by inhibiting ROS scavenging enzymes such as catalase and ascorbate peroxidases (Durner & Klessig, 1995; Zhang *et al.*, 2016). More recently, SA was proposed as a retrograde signal generated by impaired PSII proteostasis (Duan *et al.*, 2019), although whether biotic stress leads to sufficient accumulation of photodamaged proteins to instigate SA retrograde signalling remains to be demonstrated.

2. Jasmonates

Classically, jasmonates are associated with core biotrophic pathogen virulence strategies to suppress SA signalling. JA also acts synergistically with ethylene in defence against necrotrophic pathogens and ABA during herbivory (Robert-Seilaniantz *et al.*, 2011; Zhang *et al.*, 2017; Yang *et al.*, 2019). Linolenic and linoleic acid, derived from chloroplast galactolipids, provide the 18-carbon fatty acid substrate which is oxidized at the C-13 position by chloroplast lipoxygenase then cyclized to 12-oxo-phytodienoic acid (OPDA) via the consecutive activities of allene oxide synthase and allene oxide cyclase. OPDA is exported to the peroxisome where it is converted, *via* a series of beta oxidation steps, to JA which is conjugated to isoleucine to form bioactive JA-Ile. JA may undergo alternative modifications, although biological understanding of their significance is currently limited (Wasternack & Hause, 2013). With a predominant focus on jasmonate antagonism of biotrophic defences, it is often overlooked that jasmonates are also produced *de novo* during ETI (Andersson *et al.*, 2006; Zoeller *et al.*, 2012) and have been implicated in both SAR (Truman *et al.*, 2007) and induced systemic resistance (ISR) (van Wees *et al.*, 2000).

3. The role of ABA in repressing chloroplast immunity

While early studies revealed that ABA treatment suppressed resistance to biotrophic and hemibiotrophic bacterial, fungal and oomycete pathogens (Henfling *et al.*, 1980; Mohr & Cahill, 2003), it was not until subsequent, transcriptomic and genetic studies with ABA biosynthetic and signalling mutants demonstrating that pathogens hijack host ABA signalling to promote virulence that ABA became universally recognized as a key player in suppression of biotrophic immunity. *De novo* ABA synthesis induced by virulent *Pst* is remarkably rapid, occurring within 6 h of challenge, significantly preceding bacterial multiplication (de Torres-Zabala *et al.*, 2007, 2009). Pathogen-induced ABA requires transcriptional upregulation of genes encoding the chloroplast-localized 9-*cis*-epoxycarotenoid dioxygenase (NCED) and cytosolic abscisic aldehyde oxidase (AAO) – key enzymes in the final steps of ABA biosynthesis (Truman *et al.*, 2006; de Torres-Zabala *et al.*, 2007, 2009; Peng *et al.*, 2019). Concomitantly, transcripts encoding protein phosphatase 2Cs (PP2C), negative regulators of ABA signalling, are suppressed (Truman *et al.*, 2006) (see Fig. 4c later for a summary).

Carotenoid intermediates provide the precursors for ABA biosynthesis. Zeaxanthin, derived from β-carotene – whose oxidation products are themselves potential chloroplast signalling molecules (reviewed by Havaux, 2014) – is converted to violaxanthin, and then via *trans*-neoxanthin into 9′-*cis*-neoxanthin and 9′-*cis*-violaxanthin. These substrates are converted by NCED to the 15-carbon xanthoxin which is transported to the cytosol where it is converted into abscisic aldehyde and finally to ABA via AAO (Seo & Koshiba, 2002). *De novo* ABA induced by *Pst* is proposed to suppress PTI-induced cROS (de Torres Zabala *et al.*, 2015) as well as antagonizing later SA signalling (de Torres Zabala *et al.*, 2009). ABA biosynthetic mutants (*aao3*) are more resistant to *Pst* and other biotrophic pathogens. Notably, pretreatment with ABA abolished PTI, enhanced the decrease of F_v/F_m (Fig. 1e) and, analogous to ABA suppression of ROS in imbibed seeds (Ye *et al.*, 2012), induced ROS (de Torres Zabala *et al.*, 2015). As ABA can repress transcription of many plastid genes through PP2C-dependent activation of nuclear genes (Yamburenko *et al.*, 2015), the recent demonstration that *Xanthomonas* effectors of both rice and *Arabidopsis* pathogens promote virulence by suppressing transcripts encoding chloroplast-localized PP2Cs (Akimoto-Tomiyama *et al.*, 2018) reinforces the complex role of ABA in effector modulation of chloroplast immunity.

IV. cROS in immunity and insights from disruption of chloroplast components

Specificity in ROS signalling is achieved via the spatiotemporal control of production and scavenging at different organellar and

subcellular locations. During plant defence, recognition of PAMPs by PRRs activates plasma membrane-localized NADPH oxidase (Zhou *et al.*, 2019) and apoplastic type III peroxidases (Daudi *et al.*, 2012) generating, within minutes, a rapid burst of H_2O_2 comprising synthesis of short-lived superoxide and its more stable dismutation product hydrogen peroxide in the apoplast (Smirnoff & Arnaud, 2019). Hydrogen peroxide can enter the cytosol *via* plasma membrane aquaporins (Rodrigues *et al.*, 2017). ROS are also produced in organelles by oxygen reduction during electron transport and by oxidase enzymes in peroxisomes (Asada, 2006; Mullineaux *et al.*, 2018; Waszczak *et al.*, 2018; Smirnoff & Arnaud, 2019). The prominent routes for cROS generation are oxygen photoreduction at PSI (Mehler reaction) and possibly via the PSII electron acceptor plastoquinone (Dietz *et al.*, 2016; Vetoshkina *et al.*, 2017). Singlet oxygen ($^1O_2$), a highly reactive species, is formed in PSII by transfer of excitation energy from triplet-state Chl (Mullineaux *et al.*, 2018a; Dogra *et al.*, 2019) and is the major ROS involved in ETI-induced lipid peroxidation (Zoeller *et al.*, 2012).

1. PTI and cROS

As part of PTI, chloroplasts of *Arabidopsis* leaves challenged with virulent *Pst* generate reactive species (as determined by 2′7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) oxidation) which are suppressed within 4 h by *Pst* effectors (de Torres Zabala *et al.*, 2015). DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), which blocks photosynthetic electron transport between PSII and plastoquinone (Metz *et al.*, 1986), also blocks 2′7′-dichlorodihydrofluorescein oxidation, indicating that this burst is probably generated by oxygen photoreduction producing superoxide/H_2O_2 downstream of PSII (Mubarakshina *et al.*, 2010; Exposito-Rodriguez *et al.*, 2017). Interestingly, *Pst* ROS suppression coincides with a decrease in F_v/F_m (Fig. 1a,b) and photosynthesis (de Torres Zabala *et al.*, 2015) and an increase in bacterial growth (Fig. 1c) indicating that effectors (some of which are targeted to the chloroplast) interfere with critical photosynthetic components that have yet to be identified. Notably, ABA mimics DCMU application, suggesting that *Pst*-induced *de novo* ABA biosynthesis may play a key role in suppressing ROS production. Indeed, pretreatment of leaves with ABA strongly enhances the *Pst*-induced decrease in F_v/F_m and this is common to other, less virulent pathogens such as *Xanthomonas campestris* pv. *campestris* (Fig. 1e). At the same time, *Pst* (and other pathogens) suppress the expression of a large set of nuclear-encoded chloroplast genes including photosynthesis-related and antioxidant enzyme transcripts (Bilgin *et al.*, 2010; de Torres Zabala *et al.*, 2015; Su *et al.*, 2018). The signalling mechanism driving PTI-generated cROS is unclear but may involve calcium and/or retrograde signalling as discussed below.

2. cROS and ETI

The interaction between high light, phytochrome and pathogen responses has been documented (Bechtold *et al.*, 2005; Ballare, 2014). Light is required for, or enhances, ETI-triggered HR (Torres *et al.*, 2006; Nomura *et al.*, 2012). These observations suggest the interaction of cROS with photosynthesis, SA production (Chaouch *et al.*, 2010, 2012) and additionally NO (Zaninotto *et al.*, 2006; Yun *et al.*, 2011; Yun *et al.*, 2016). The development of an HR is rapid and effectively contains the pathogen. The HR is widely thought to be triggered by $^1O_2$ generation, which leads to lipid peroxidation (Havaux, 2014). Pioneering analytical studies of the temporal accumulation of oxidation products derived from unsaturated fatty acids during the HR strongly support a $^1O_2$ burst. Notably, the HR leads to an early and massive accumulation of both enzymatic and nonenzymatic chloroplast galactolipid-derived oxylipins (Andersson *et al.*, 2006; Zoeller *et al.*, 2012) with huge increases in JA measured within 5 h of infection with *PstavrRpm1* (Zoeller *et al.*, 2012). This timing is consistent with the earlier biophoton production following *PstavrRpm1* challenge (Bennett *et al.*, 2005), which is indicative of lipid oxidation (Havaux *et al.*, 2006). For example, HR in Arabidopsis inoculated with *PstavrRpm1* is enhanced by increased light intensity and associated with disruption of the PSII light harvesting complex, decreased F_v/F_m (Fig. 1f) and accumulation of the Chl catabolite phaeophorbide, a potent photosensitizer that generates $^1O_2$ (Mur *et al.*, 2010). Indeed, F_v/F_m provides a powerful readout to accurately capture and quantify the timing of specific R protein activation, before visible symptoms, as illustrated for the RPM1–AvrRpm1 and RPS4–AvrRPS4 interactions (Fig. 1f; for a recent review see also Perez-Bueno *et al.*, 2019).

Further evidence that chloroplast-sourced ROS are involved in ETI and mediated by MAPK pathways are provided by studies in *Nicotiana benthamiana* (Liu *et al.*, 2007) and *Arabidopsis* (Su *et al.*, 2018). *PstavrRpt2* (like *PstavrRpm1*, Fig. 1f) causes a much larger and earlier decrease in PSII quantum efficiency than *Pst* (Fig. 1a) (de Torres Zabala *et al.*, 2015), and a more prolonged activation of MAPKs (Su *et al.*, 2018). This response is mirrored by conditional induction of MAPKs (MPK3/6) leading to cell death. Both *PstavrRpt2* and MAPK activation increase cROS within 6 h in a light-dependent manner (consistent with biophoton generation at *c.* 7 h post-inoculation (hpi); Bennett *et al.*, 2005), accompanied by visible disruption of PSII. Comparison of apparent chloroplast-sourced ROS between PTI and ETI in this system indicates that chloroplast-targeted effectors decrease photosynthesis and suppress cROS production (de Torres Zabala *et al.*, 2015) whereas ETI involves a more aggressive effect on photosynthesis, as illustrated by rapid decreases in F_v/F_m and an increase in ROS (Su *et al.*, 2018), consistent with extensive chloroplast galactolipid oxidation recorded during early ETI (Andersson *et al.*, 2006; Zoeller *et al.*, 2012).

3. A role for cROS in systemic immunity?

The chloroplast is becoming increasingly linked to effective SAR, a process conferring broad-spectrum and lasting immunity to pathogens of diverse lifestyles (Fernandez & Burch-Smith, 2019). Classic SAR is established following successful ETI leading to the HR. Chloroplast lipids and cROS appear to be central to generation of SAR inducing signal(s) following ETI-activated HR (Wendehenne *et al.*, 2014; Shine *et al.*, 2019), supported by the SAR-deficient phenotypes of fatty acid desaturase (*sfd2*;

SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY2) mutants. The chloroplast galactolipid mutants *mgd1* and *dgd1* (*monogalactosyl synthase 1*, *digalactosyl synthase 1*), responsible for monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) synthesis respectively, function nonredundantly in initial SAR signal perception (Gao *et al.*, 2014; Shah *et al.*, 2014). As noted above, ETI-generated ROS modify fatty acids on chloroplast galactolipids leading to rapid JA accumulation (Andersson *et al.*, 2006; Zoeller *et al.*, 2012). Although JA is classically associated with suppression of SA signalling in biotrophic interactions, jasmonates have been implicated in both SAR (Truman *et al.*, 2007; Liu *et al.*, 2016) and ISR (van Wees *et al.*, 2000). While somewhat speculative, interesting parallels are emerging between the role of ROS in signalling pathways that regulate SAR and systemic acquired acclimation (SAA) and wounding, where a wave of ROS signalling appears to be a common early mediator of systemic signalling responses although on different time scales (Zandalinas *et al.*, 2019).

V. Immunity insights from perturbation of chloroplast metabolism and cROS production

The following sections describe components of the photosynthetic electron transport system and the main sources of ROS occurring in the chloroplast. The reader is referred to the schematic in Fig. 2 for context. The removal of H_2O_2 in chloroplasts is carried out by a diverse set of enzymes providing robustness to PTI. These include ascorbate peroxidases localized to the stroma or attached to the thylakoid membrane along with glutathione and associated enzymes to regenerate oxidized ascorbate: glutathione peroxidase-like and peroxiredoxins (Smirnoff & Arnaud, 2019) (Fig. 2). Oxidized peroxiredoxins are regenerated by thioredoxin with involvement of NADPH-dependent thioredoxin reductase C (NTRC) (Perez-Ruiz *et al.*, 2017). While mutants of individual peroxiredoxins (Prx) in Arabidopsis (there are four chloroplast Prx isoforms in *Arabidopsis*; Tripathi *et al.*, 2009), have normal *Pst* responses, an NTRC mutant (*ntrc*) shows increased cell death and increased peroxide production as determined by 3,3'-diaminobenzdine staining (Ishiga *et al.*, 2011) but no difference in *Pst* growth compared to wild-type Col-0. Interestingly, the authors also showed that NTRC-silenced tomato plants showed accelerated necrotic cell death and enhanced symptom development in response to the necrotrophic soil pathogen *Sclerotinia sclerotiorum*. A similar response was elicited by nonhost *P. syringae*, although pathovars varied in specific responses (Ishiga *et al.*, 2016). Notably, these symptoms were absent in plants inoculated with a coronatine (COR)-deficient *Pst* strain, implicating a role for COR in cROS-induced disease-associated necrosis (Ishiga *et al.*, 2016). Antisense knockdown of two chloroplast GPX-like enzymes in Arabidopsis increases H_2O_2 and high light-induced SA. These plants had elevated PTI to *Pst* and *P. syringae* pv. *maculicola* (*Psm*), and more extensive HR following *PstavrRpm1*-initiated ETI (Chang *et al.*, 2009). Manipulation of chloroplast APX also impacts pathogen response. Rice lines overexpressing thylakoid membrane-bound APX exhibited increased initial tolerance to rice bacterial blight conferred by *Xanthomonas oryzae* pv. *oryzae*, whereas RNAi lines were more susceptible, and this was correlated with H_2O_2 levels, presumably chloroplast-derived (Jiang *et al.*, 2016). In *Arabidopsis*, conditional silencing of thylakoid-bound APX showed that accumulation of chloroplastic H_2O_2 triggered retrograde signalling leading to induction of nuclear-encoded pathogen defence genes in the absence of any pathogen challenge (Maruta *et al.*, 2012). While not confined to chloroplasts, the concentration of the antioxidants ascorbate and glutathione, which are involved in H_2O_2 removal and redox regulation, influence pathogen responses. Ascorbate-deficient mutants have increased H_2O_2, PR levels, camalexin and SA accumulation and have increased basal resistance to *Pst* and the oomycete *Hyaloperonospora* (Barth *et al.*, 2004; Pavet *et al.*, 2005; Colville & Smirnoff, 2008; Mukherjee *et al.*, 2010). Consistent with these observations, glutathione-deficient mutants have decreased resistance to *PstavrRpm1* (Ball *et al.*, 2004; Parisy *et al.*, 2007).

Expressing the cyanobacterial electron transport protein flavodoxin in tobacco chloroplasts improves robustness of photosynthesis to various stresses including methyl viologen (MV: a redox cycling compound that generates superoxide at PSI) and high light. This appears to be associated with decreased cROS production (Tognetti *et al.*, 2006) and altered pathogen responses (Zurbriggen *et al.*, 2009; Rossi *et al.*, 2017). The reason that flavodoxin, which has a flavin cofactor, improves stress resistance and decreases cROS production is not immediately apparent. It functionally replaces the plant PSI electron acceptor ferredoxin (Tognetti *et al.*, 2006) which has a 2Fe–2S reaction centre. One possibility is that electron transport through flavodoxin decreases oxygen photoreduction at PSI (the Mehler reaction) (Fig. 2). Alternatively, because Fe–S proteins are a target for superoxide and H_2O_2, which can demetallate them (Imlay, 2013), chloroplastic ferredoxin may be sensitive to inactivation by ROS. Indeed, superoxide inactivates spinach ferredoxin (Fisher *et al.*, 2016), consistent with the marked increase in resistance to MV (Tognetti *et al.*, 2006). This may account for the significant reduction in localized cell death induced by the nonhost pathogen *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) in flavodoxin-expressing tobacco leaves, which was associated with decreased cROS production (Zurbriggen *et al.*, 2009). Similarly, infection of flavodoxin-overexpressing tobacco with the necrotrophic fungus *Botrytis cinerea* significantly restricted hyphal growth, lesion development, *Pathogenesis Related* (*PR*) gene expression and phytoalexin accumulation (Rossi *et al.*, 2017). Expression of flavodoxin in *Arabidopsis* chloroplasts decreases ROS production and disassembly of PSII in response to *PstavrRpt2*, attenuating ETI (Su *et al.*, 2018). These studies highlight a central role for cROS in effective PTI and ETI. A mutant in the main chloroplast ferredoxin (*fd2*; Fig. 2) exhibiting altered pathogen responses provides additional evidence linking electron transport from PSI with PTI (Wang *et al.*, 2018). *fd2* was more susceptible to *Pst*, possibly as a direct result of the elevated JA observed. By contrast, ETI elicited by AvrRpt2 was stronger, with twice as much H_2O_2 generation. This result is part of a growing body of evidence for possible photosystem-specific roles for ROS generation during ETI and PTI, with ROS generated by ETI being primarily derived from PSII whereas PTI may generally require electron transport to PSI, which is compromised in *fd2* plants. This is also consistent

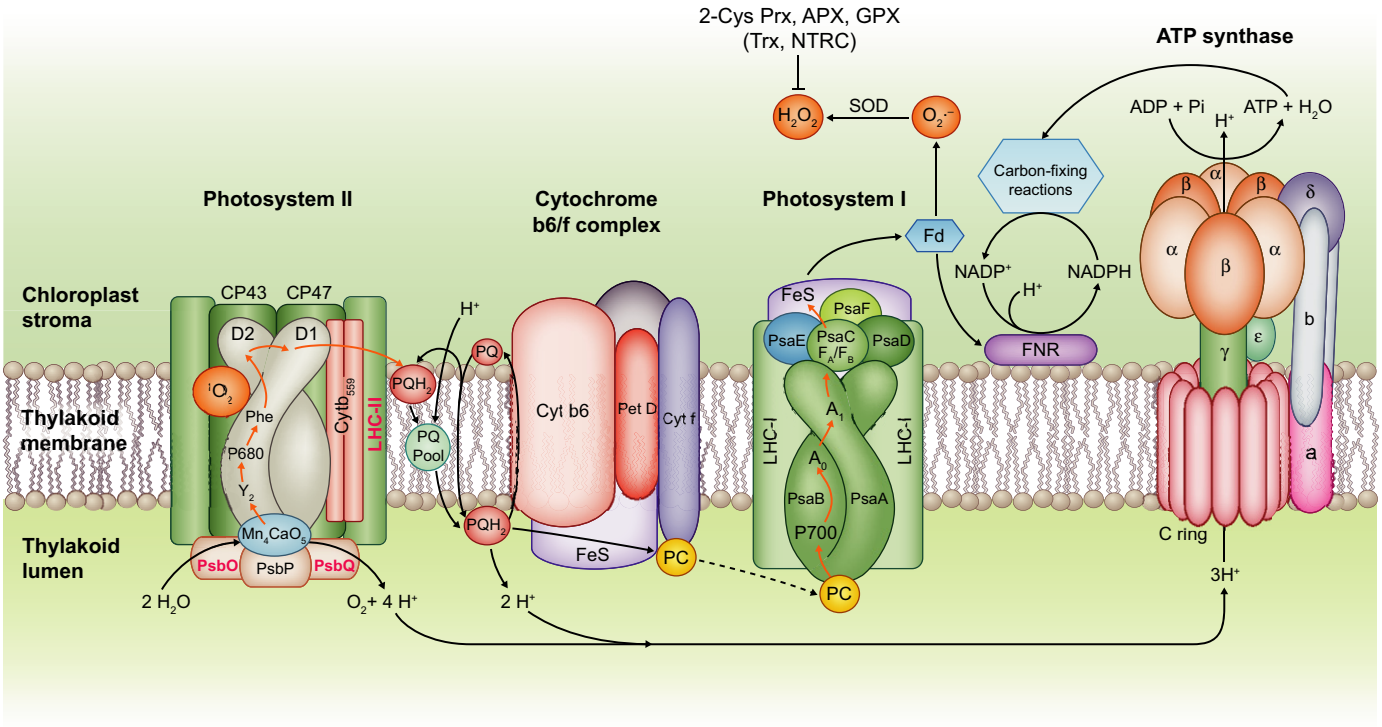

Fig. 2 An outline of the photosynthetic electron transport system showing the main sources of reactive oxygen species (ROS; singlet oxygen ($^1O_2$), superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2)) at photosystems I and II. Proteins that are validated effector targets are shown in red. APX, ascorbate peroxidase; 2-Cys Prx, 2-cysteine peroxiredoxin; Fd, ferredoxin; FNR, ferredoxin-NADPH reductase; GPX, glutathione peroxide-like; NTRC, NADPH-dependent thioredoxin reductase; PQ, plastoquinone; PC, plastocyanin; SOD, superoxide dismutase; Trx, thioredoxin.

with PSI being the source of H_2O_2 for PTI (de Torres Zabala *et al.*, 2015).

In conclusion, various lines of evidence show that cROS is induced by PTI and ETI, and we speculate that H_2O_2 derived from PSI may be the primary ROS underpinning PTI whereas ETI elicits rapid accumulation of $^1O_2$ (Fig. 2). The intensity and duration of the response, and hence the eventual pathogenic outcome is dictated by a complex interaction, its outcome being dictated by the specific pathogen virulence strategy and host resistance protein complement. Higher H_2O_2 levels can improve basal immunity, but effectors collaborate to directly or indirectly repress cROS production, probably by inhibiting electron transport to PSI. By contrast, and somewhat counterintuitively, ETI appears to elicit an extensive disruption of photosynthesis, including breakdown of PSII leading to greater ROS production and the HR. This is likely to be driven by $^1O_2$. However, at this point a fuller understanding of these mechanisms is limited by the poor specificity of the ROS assays (Smirnoff & Arnaud, 2019). New genetically encoded reporters (Nietzel *et al.*, 2019) and nanosensors (Lew *et al.*, 2020) offer better specificity and temporal spatial resolution to better dissect these processes.

VI. Direct targeting of pathogen effectors to the chloroplast

The previous sections show that pathogen effectors modulate chloroplast function, either directly or indirectly, which implies that effectors may themselves localize to the chloroplast and directly interact with chloroplast-located targets. Here we summarize direct and indirect experimental evidence for effector localization to the chloroplast.

1. Bacterial effectors

Evidence for physical targeting of chloroplasts by bacterial effectors did not emerge until the mid-2000s (Jelenska *et al.*, 2007; Lee *et al.*, 2008) yet remarkably and more than 10 of *Pseudomonas syringae*'s core effector repertoire of 30–40 proteins have been predicted or experimentally shown to localize to the chloroplast (Table 1). More recently, a number of effector proteins from *Ralstonia solanocerarum* have been shown to localize to the chloroplast (Table 1), although knowledge of their host targets is limited (Jelenska *et al.*, 2007; Lee *et al.*, 2008; Rodriguez-Herva *et al.*, 2012).

Pseudomonas syringae The recently described *P. syringae* pan genome (Laflamme *et al.*, 2020) has provided a rich resource to further expand our knowledge of effectors targeted to the chloroplast.

HopI1 One of the first bacterial effectors found to target the chloroplast was HopI1 from *Psm.* HopI1 has a redundant chloroplast-targeting sequence and contains P/Q-rich repeats (to facilitate protein folding) and a J domain, through which it directly

Table 1 Pathogen effectors predicted to be chloroplast localised.

Effector	Origin species	Pathovar	Localisation	Experimental method for localisation	Chloroplastic Interacting partner	Interacting partner identification	ETI/ETS	Reference
Bacteria								
HopI1	*Pseudomonas syringae*	*maculicola*	Chloroplast	Transgenic Arabidopsis, chloroplast fract, c onfocal in transient Nb	HSP70	Yeast complementation	ETS	Jelenska *et al.* (2007)
AvrRps4	*P. syringae*	*pisi*	Chloroplast, Nucleus Cytoplasm	GFP fusion transient in Nb			Extachloro-plastic ETI	Li *et al.* (2014)
HopK1	*P. syringae*	*tomato*	Chloroplast, Nucleus Cytoplasm	GFP fusion transient in Nb			ETS	Li *et al.* (2014)
HopO1-2	*P. syringae*	*tomato*	Chloroplast	Import assay (isolated pea chloroplasts)			ETS	de Torres Zabala *et al.* (2015)
HopR1	*P. syringae*	*tomato*	Chloroplast	Import assay (isolated pea chloroplasts)	PTF1, CBSX2	Y2H	ETI -transient expression in Nb	Mukhtar *et al.* (2011), de Torres Zabala *et al.* (2015)
HopN1	*P. syringae*	*tomato*	Chloroplast	GFP fusion transient in Nb	PsbQ	*in vitro* pull-down	ETS	Rodriguez-Herva *et al.* (2012)
HopBB1	*P. syringae*	*tomato*	Chloroplast	Predicted – *in silico*	PTF1	Y2H		Mukhtar *et al.* (2011)
HopM1	*P. syringae*	*actinidiae*	Chloroplast	YFP fusion transient in Nb				Choi *et al.* (2017)
HopU1	*P. syringae*	*tomato*			RBP31 plus two additional chloroplast RNA-binding proteins	Y2H		Mukhtar *et al.* (2011)
HopZ1	*P. syringae*	*tomato*			AT4G39050, AT3G11590, AT3G07780	Y2H		Mukhtar *et al.* (2011)
RipAL	*Ralstonia solanacearum*		Chloroplast	GFP fusion transient in Nb				Nakano & Mukaihara (2018)
RipAD	*R. solanacearum*		Chloroplast	YFP fusion transient in Nb				Jeon *et al.* (2020)
RipG3, RipG7	*R. solanacearum*				Nbcab13, NbrbcX, NbrbcS	Y2H		Dahal *et al.* (2018)
Fungi								
ToxA	*Pyrenophora tritici-repentis*		Chloroplast	GFP fusion transient in Nb	ToxABP1 Thf1	Y2H	ETS	Manning *et al.* (2007), Sperschneider *et al.* (2017)
CTP1, CTP2, MLP124111	*Melampsora larici-populina*		Chloroplast	GFP fusion transient in Nb				Petre *et al.* (2015), Petre *et al.* (2016)
CTP3	*M. lini*		Chloroplast	GFP fusion transient in Nb				Petre *et al.* (2015), Petre *et al.* (2016)

Table 1 (Continued)

Effector	Origin species	Pathovar	Localisation	Experimental method for localisation	Chloroplastic Interacting partner	Interacting partner identification	ETI/ETS	Reference
PST03196, PST18220	*Puccinia striiformis f. sp. tritici*		Chloroplast	GFP fusion transient in Nb				Petre *et al.* (2016a,b)
PGTG_00164, PGTG_06076	*Puccinia graminis f. sp. tritici*		Chloroplast	GFP fusion transient in Nb				Sperschneider *et al.* (2017)
SsITL	*Sclerotinia sclerotiorium*		Chloroplast		CAS (calcium-sensing)			Tang *et al.* (2020)
Oomycete PhRXLR-C20 PhRXLR-C27*	*Plasmopara halstedi*		Chloroplast	YFP fusion transient in Nb				Pecrix *et al.* (2019)
PvRXLR54, PvRXLR61, PvRXLR86, PvRXLR161	*Plasmopara viticola*		Chloroplast, nucleus, mitochondria	Experimental				Liu *et al.* (2018)

*Chloroplast associated; CBS, CBS Domain containing protein; HSP70 Heat shock 70 protein; Nb, *Nicotiana benthamiana*; PTF1, PHYTOCHROME AND FLOWERING TIME; Nbrbc, ribulose bisphosphate carboxylase small chain from *N. benthamiana*; Nbcab13, chlorophyll a-b binding protein 13 from *N. benthamiana*; Thf1, Thylakoid formation 1.

binds to and affects the activity and/or specificity of chloroplast-associated cytosolic Heat shock protein 70 (Hsp70). While *in planta* interaction with the Hsp70 chloroplast isomer has yet to be demonstrated, HopI1 induces altered thylakoid structure and reduced SA accumulation (Jelenska *et al.*, 2007), although how HopI1 enters the chloroplast remains to be determined.

HopN1 HopN1 suppresses ROS accumulation, callose deposition and HR cell death (López-Solanilla *et al.*, 2004; Rodriguez-Herva *et al.*, 2012), these activities being dependent on its cysteine protease activity. HopN1 localizes to the thylakoid membrane, interacting with and degrading PsbQ from PSII, reducing oxygen production, electron transport and attenuating cROS. Collectively, these studies have shown that PsbQ quantitatively contributed to both PTI and nonhost HR.

AvrRps4/HopK1 AvrRps4 is more commonly associated with triggering ETI when recognized by RPS4 in *A. thaliana*. However, AvrRps4 localizes to both the nucleus and the chloroplast and has high N-terminal sequence homology to another effector protein, HopK1. Both AvrRps4 and HopK1 target the chloroplast via a cleavable transit peptide (Li *et al.*, 2014) and their chloroplast localization is required to suppress the classical PTI responses, ROS production and callose deposition, and to enhance bacterial growth. The generation of combinations of chimeric effectors between C- and N-terminal domains of AvrRps4 and HopK1 demonstrated that AvrRps4 contributes to bacterial virulence in *Pst* lacking HopK1, although the chloroplast targets of these effectors remain to be determined. $HopK1^{N}$-$AvrRps4^{C}$ but not $AvrRps4^{N}$-$HopK1^{C}$ chimeras induced a strong HR delivered through *Pseudomonas fluorescens* (Halane *et al.*, 2018). However, $AvrRps4^{N}$ not only directly interacted with EDS1 but also contributed to bacterial virulence in *Pst* lacking HopK1, establishing AvrRps4 as an evolved bipartite effector with dual nuclear and chloroplast functions (Halane *et al.*, 2018).

HopM1 *Pst* HopM1 localizes to the *trans*-Golgi network where it interacts with the ADP-ribosylation factor guanine nucleotide exchange factor, AtMIN7, to suppress vesicle-trafficking (Nomura *et al.*, 2011). However, HopM1 from *P. syringae* pv. *actinidiae*, with 67% amino acid identity to *Pst* HopM1, localizes to the chloroplast (Choi *et al.*, 2017), suggesting an intriguing evolution of alternative functions for these proteins.

Of the remaining *P. syringae* effectors that are known to localize to the chloroplast, the predicted ADP-ribosyl transferase HopO1-2 and HopR1 translocate into isolated chloroplasts (de Torres Zabala *et al.*, 2015) although further functional insight is lacking. HopR1 and HopO1-2 were amongst a number of effectors identified to interact with predicted chloroplast-localized proteins in yeast two-hybrid screens, including HopU1, HopZ1, HopW1 and HopBB1 (Lee *et al.*, 2008; Mukhtar *et al.*, 2011).

Other bacterial effectors Evidence for effectors targeting to the chloroplast is emerging from other bacterial pathogens. The chloroplastic phospholipase A1 RipAL (*Ralstonia*-injected proteins) from *Ralstonia solanacearum* (Nakano & Mukaihara, 2018)

shares homology with DEFECTIVE IN ANTHER DEHISCENCE1 (Ishiguro *et al.*, 2001), which catalyses the release of linoleic acid, a critical precursor of JA biosynthesis, from chloroplast membranes. RipAL localizes to the chloroplast and wild type, but not a lipase active site mutant, suppressed PTI in *N. benthamiana* via enhanced JA signalling and JA/JA-isoleucine content, with a concomitant decrease in SA and associated SA-signalling genes (Nakano & Mukaihara, 2018). The F-box domain RipG effector family comprises seven members, of which RipG3 and RipG7 interact with chloroplast proteins – possible targets for ubiquitination and proteasomal degradation (Dahal *et al.*, 2018). RipAD is also localized to chloroplasts, although its host target(s) remain unknown (Jeon *et al.*, 2020). Notably, both RipAL and RipAD interfere with flg22-triggered ROS production presumably from the chloroplast (Nakano & Mukaihara, 2018; Jeon *et al.*, 2020).

2. Effectors from filamentous pathogens

Chloroplast-localized effector proteins from fungi and oomycetes are now being identified, indicating that filamentous pathogens have also evolved to target the chloroplast (Table 1).

Rusts Transient expression in *N. benthamiana* has localized eight effector proteins from rusts (Table 1) (Petre *et al.*, 2015; Petre *et al.*, 2016; Sperschneider *et al.*, 2017). Notably, the program LOCALISER has proved useful for *in silico* prediction of chloroplast and other cellular effector addresses (Sperschneider *et al.*, 2017), identifying a further two chloroplast-localized effectors from the biotrophic rust *Puccinia graminis* f. sp. *tritici*, PGTG_00164 and PGTG_06076, which were experimentally validated.

Given the dearth of experimentally validated chloroplast-localized effector proteins from other fungi, this may reflect a rust virulence strategy or lack of experimental endeavour.

Oomcyete Oomcyete effectors are largely of the 'RXLR' class. RXLRs are defined by a secretion signal peptide followed by a conserved N-terminal domain comprising the RXLR (Arg–Xaa–Leu–Arg) consensus sequence, where X is any amino acid that shares a conserved structural fold (Win *et al.*, 2012). A high-throughput screen of 83 candidate RXLR effectors of the obligate biotrophic oomycete *Plasmopara viticola* (Liu *et al.*, 2018) identified four effectors localized to the chloroplast (Table 1). Only one contained a cleavable N-terminal transit peptide and was specifically targeted to the chloroplast, PvRXLR86, whereas the others had multiple organellular addresses (Liu *et al.*, 2018). PvRXLR61 and PvRXLR161 localized to the chloroplast and nuclei whereas PvRXLR54 additionally targeted the mitochondria (Liu *et al.*, 2018). A chloroplast-localized effector was also identified from the related sunflower powdery mildew, *Plasmopara halstedii*. PhRXLR-C20, expressed during pathogen colonization, was observed in the chloroplast and stromules (Pecrix *et al.*, 2019). Notably, PhRXLR-C27 targeted plastid-associated membranes (Pecrix *et al.*, 2019). The host targets of these two effectors remain unknown.

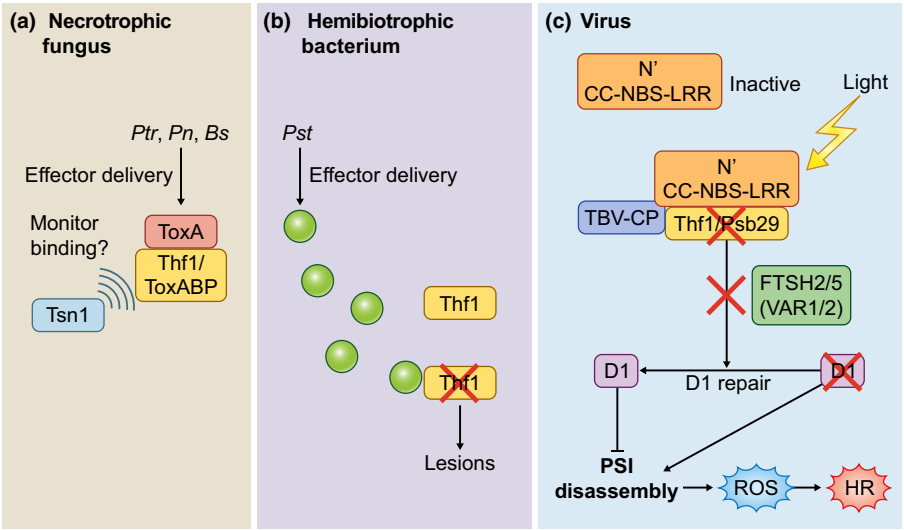

Fig. 3 Convergent targeting of Thylakoid formation 1 (Thf1), a negative regulator of cell death, by diverse pathogens. Thf1 plays an important role in photosystem II (PSII) – light harvesting complex II dynamics and is targeted by necrotrophs, biotrophs and viruses. (a) The effector protein ToxA found in a variety of necrotrophic wheat fungal pathogens, *Parastagonospora nodorum* (*Pn*), *Pyrenophora tritici-repentis* (*Ptr*) and *Bipolaris sorokiniana* (*Bs*), targets the wheat Thf1 orthologue, ToxA Binding Protein 1 (ToxABP), inducing necrosis via ROS accumulation through reduction in PSI and PSII protein complex abundance. The wheat sensitivity protein, Tsn1, is required for ToxA-dependent necrosis and may monitor binding of ToxA to ToxABP1. (b) The hemibiotrophic bacterium *Pseudomonas syringae* pv. *tomato* (*Pst*) delivers effectors (yellow circles) which appear to disrupt Thf1 function, again leading to enhanced lesion formation, although it remains to be determined whether this is by direct or indirect interaction. (c) *The Tobamovirus* (TBV) N′ virus resistance protein, belongs to the conserved Solanaceae I2 class of CC-NBS-LRR resistance protein.that also confersresistance to *Phytophthora* and *Fusarium* sp.TBV's CC domain physically targets and destabilizes TBV-coat protein in a light-dependent manner to enhance resistance. Based on analogy to the cyanobacterium *Synechocystis* Thf1 orthologue, Psb29, Thf1 destabilization affects accumulation of the FtsH ATP-dependent zinc metalloproteases, FTSH2 and FTSH5 (also known as *VAR2* and *VAR1* respectively), which are involved in the selective degradation of PSII subunits, such as D1 during PS repair. This would lead to PSII disassembly and increased ROS production.

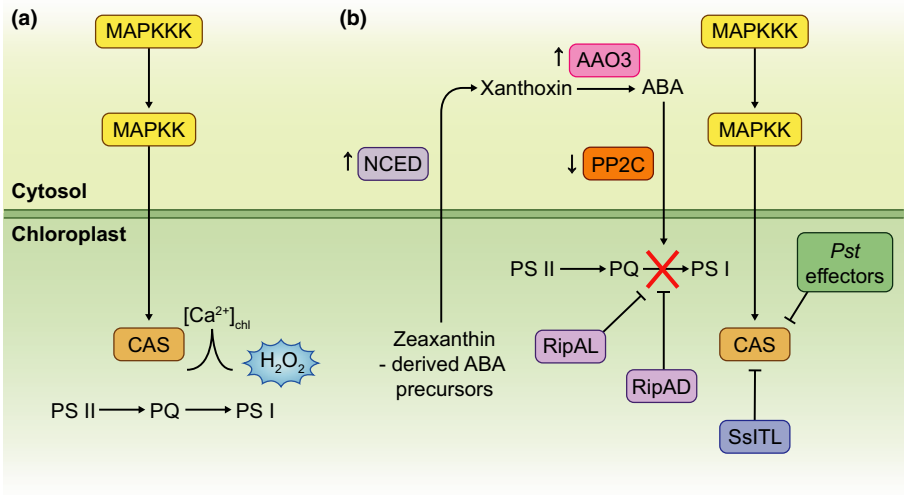

Fig. 4 Modulation of calcium and reactive oxygen species (ROS) during suppression of PAMP-triggered immunity (PTI). (a) PTI initiates stromal Ca^{2+} spikes *via* mitogen-activated protein kinase (MAPK) activation of the chloroplast-localized CAS (calcium sensing protein), and these changes in $[Ca^{2+}]_{cp}$ are necessary for callose deposition and stomatal closure. *cas* mutants are compromised in resistance to both virulent and avirulent *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). (b) The integrin-like effector SsITL (blue) from the soil fungal pathogen *Sclerotinia sclerotiorum* directly targets CAS to suppress immunity. Virulent *Pst* attenuates cROS by rapid induction of *de novo* ABA synthesis. *Pst* effector delivery rapidly induces expression of *NCED3* and *AAO3*, encoding key enzymes in ABA biosynthesis while concomitantly suppressing expression of the *PP2C*s, encoding negative regulators of ABA signalling. ABA application suppresses PTI-induced chloroplastic ROS generation and enhances effector suppression of F_v/F_m. RipAL and RipAD, *Ralstonia solanacearum* effectors, also target the chloroplast and suppress cROS, although the mechanism remains to be determined. CAS, Ca^{2+}-sensing protein; NCED, 9-*cis*-epoxycarotenoid dioxygenase; AAO3, abscisic aldehyde oxidase 3; PP2C, protein phosphatase 2C; Rip, *Ralstonia*-injected protein; PQ, plastocyanin; SsITL, *Sclerotinia sclerotiorum* integrin-like protein; PS, photosystem; PQ, plastocyanin (see Fig. 2 for more details).

Necrophic fungal effectors ToxA, a 178 amino acid secreted necrotrophic effector protein was first isolated from the fungus *Pyrenophora tritici-repentis* (Sarma *et al.*, 2005) and was more recently identified in *Parastagonospora nodorum* and *Bipolaris sorokiniana* (McDonald *et al.*, 2017). ToxA targets the chloroplast ToxA Binding Protein 1 (ToxABP1), inducing ROS accumulation through decrease in PSI and PSII protein complex abundance (Manning *et al.*, 2007; Faris *et al.*, 2010). The sensitivity in wheat to ToxA is governed by the *Tsn1* locus, encoding classical nucleotide binding, leucine rich repeat disease resistance proteins, suggesting these may monitor ToxA activity. The severity of necrosis can be restricted by preventing ROS accumulation or silencing ToxABP1 (Manning *et al.*, 2007). The *A. thaliana* homologue of the wheat ToxABP1, known as Thylakoid formation 1 (Thf1), is also targeted by multiple pathogens (see below), suggesting convergent evolution of effector targets. The *S. sclerotiorum* effector SsITL has recently been shown to localize to the chloroplast and interact with the chloroplast-localized calcium-sensing receptor (CAS, see below) (Tang *et al.*, 2020). The interaction of SsITL with CAS interferes with the SA signalling pathway to reduce SA accumulation during early infection while overexpression of CAS increased resistance to *S. sclerotiorum* (Tang *et al.*, 2020).

3. Convergent targeting of Thf1, a negative regulator of cell death, by diverse pathogens

Aside from being a target of ToxA, chloroplast-localized Thf1 is involved in a range of host–microbe interactions (necrotrophic, biotrophic, viral), mediating both PTI and ETI (Fig. 3). Thf1 is an orthologue of ToxABP1 which binds ToxA (see above, Fig. 3a) and plays a central role in controlling PSII–light-harvesting complex II (LHCII) dynamics during dark-induced senescence and light acclimation (Huang *et al.*, 2013). It has also been linked to DC3000 virulence and virus infection (Fig. 3b,c). Both virus-induced gene-silenced *SlALC*, the tomato Thf1 orthologue, and *Arabidopsis thf1* mutants exhibited accelerated lesion formation upon DC3000 challenge, and *SlALC1* chloroplast localization was affected by coronatine (Wangdi *et al.*, 2010). Interestingly, Thf1 was additionally identified as an interactor with the CC domain of the Solanaceae I2-like class of CC-NLRs (Ori *et al.*, 1997), which provide immunity against a range of pathogens including *Fusarium oxysporum* f. sp. *lycopersici* (Hamel *et al.*, 2016), *Phytophthora infestans* in potato (Huang *et al.*, 2005) and *Tobamovirus* coat protein in pepper (Tomita *et al.*, 2011). Using N′, an I2 CC-NLR which recognizes *Tobamoviruses* coat protein (Hamel *et al.*, 2016) demonstrated that Thf1 functions as a negative regulator of cell death, and activation of N′ results in the destabilization of Thf1 in a light-dependent manner (Fig. 3c). Notably, like the TMV N protein interaction with chloroplast-localized NRIP protein (see below (Caplan *et al.*, 2008)), the N′–Thf1 interaction appears to take place in the cytosol. Possible insight into how Thf1 destabilization impacts chloroplast immunity is provided by the demonstration that a cyanobacterial Thf1 homologue Psb29 is required for the accumulation of the FtsH ATP-dependent zinc metalloproteases, which function in selective degradation of PSII subunits during repair (Beckova *et al.*, 2017). Normally, inactivation of PSII is restored through a repair cycle replacing damaged

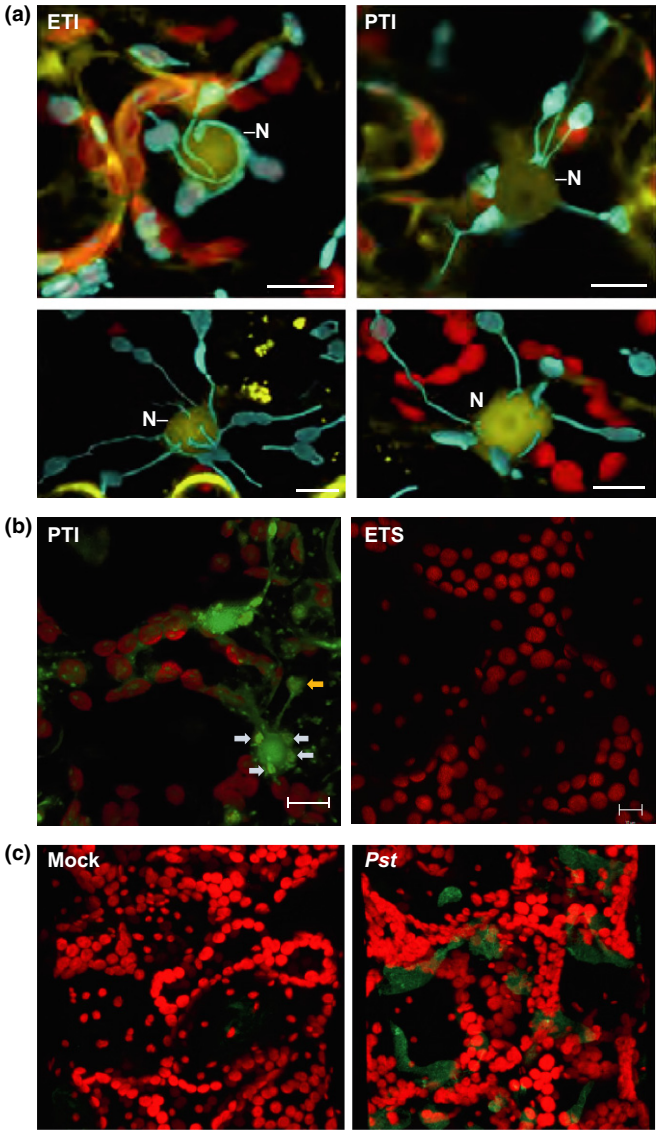

Fig. 5 Physical responses of chloroplasts to pathogen infection. Stromule formation is common to both ETI and PTI, possibly providing a conduit of physical retrograde communication. (a) Confocal micrographs of tobacco N protein TMV p50-mediated ETI with chloroplasts visualized in N-containing NRIP1-Cerulean plants. Upper left: stromules wrapped around nuclei (maximum-intensity projection of a z stack). Upper right: direct connection to the nucleus of clusters of stromule tips (single z stack plane). Lower panels: nuclei with a mixture of tip or surrounding stromule connections (transparent projections of z stacks). Bars, 10 μm (from Caplan *et al*., 2015). (b) Confocal images of reactive oxygen species (visualized by 2′,7′-dichlorodihydrofluorescein diacetate [H_2DCFDA] staining) in nucleus and chloroplasts of leaf cells challenged with the nonvirulent *Pseudomonas syringae* pv. *tomato* (*Pst*) *hrpA* mutant eliciting PAMP-triggered immunity (PTI; left panel, bar 20 μm) or virulent *Pst* capturing effector-triggered susceptibility (ETS; right panel, bar 10 μm) visualized *c.* 5 h post-inoculation. White arrows denote chloroplasts sitting on the nucleus – both organelles show strong H_2DCFDA staining. Yellow arrow represents an H_2DCFDA F-stained chloroplast whose stromule is associated with the nucleus. Red fluorescence corresponds to Chl and green channel to the H_2DCFDA signal. Bars, 10 μm. (c) Compared with mock challenge (left panel) chloroplast aggregation is seen during *Pst* ETS in *A. thaliana* (18 h post-infection (hpi)). Red fluorescence signal is derived from Chl and green fluorescence from *Pst* labelled with GFP (adapted from Hutt *et al*., 2014).

protein subunits, mainly the D1 reaction centre subunit, with functional copies. Damaged D1 repair is usually mediated through proteolysis by members of the Arabidopsis FTSH family. Thf1 is required for normal accumulation of FTSH2 and FTSH5 (also known as *VAR2* and *VAR1* respectively; Wu *et al*., 2013). Thus N′ destabilization of Thf1 would diminish FTSH2/5 levels, impacting PSII repair, and lead to the production of ROS and presumably HR cell death (Fig. 3c).

4. Getting the message across: is calcium signalling involved?

There are common and distinct roles for ROS and calcium signalling in activating and uncoupling chloroplast immunity. Calcium signalling, like ROS signalling, is probably via a propagative wave, initiated at the plasma membrane upon PRR activation and transmitting to the chloroplast and nucleus, although current knowledge of this remains sparse. Twenty years ago, rapid transient cytosolic calcium (Ca^{2+}_{cyt}) increases in response to PTI (*Pst*, *PsthrpA* and *Pst avrRpm1* challenges) were recorded using the calcium-sensitive reporter aequorin (Grant *et al*., 2000). *PstavrRpm1* (ETI) elicited an additional slow, sustained increase in Ca^{2+}_{cyt}, yet it is still unclear whether this is a signal perceived by other organelles, or indicative of loss of Ca^{2+} homeostasis coincident with HR development.

A role for calcium in establishment of chloroplast immunity is evidenced from studies on the thylakoid-membrane-localized Ca^{2+}-sensing protein (CAS), which generates stromal Ca^{2+} spikes via Ca^{2+} release from thylakoid membranes (Fig. 4a). The *cas-1* mutant was strongly compromised in resistance to virulent and avirulent *Pst* (Fig. 4b). Additionally, classical PTI responses such as callose deposition and stomatal closure were attenuated in *cas-1*. Biochemical characterization of *CAS*-silenced *N. benthamiana* plants positioned CAS downstream of activated MAPK signalling cascades and upstream of ROS signalling (Nomura *et al*., 2012). Recently, the *S. sclerotiorum* integrin-like effector *Ss*ITL was shown to directly target CAS to suppress immunity (Fig. 4b) (Tang *et al*., 2020). Ss*ITL*-expressing transgenic plants were more susceptible and *CAS* overexpression enhanced resistance to *S. sclerotiorum*, consistent with the previously reported role of *Ss*ITL in suppression of JA/ethylene signalling (Zhu *et al*., 2013). Thus, stromal calcium signalling appears important in mediating broad-spectrum immunity.

VII. Cellular reorganization during infection, stromules and perinuclear chloroplast movement

Subcellular reorganization is well documented during plant–pathogen interactions. In addition to the generation of specialized interfaces between plant cells and invading pathogens (e.g. the extrahaustorial membrane (EHM) and biotrophic interfacial complex (BIC)), cellular components are recruited to sites of infection, often mediated by actin microfilaments or microtubules, as recently reviewed (Park *et al*., 2018b; Boevink *et al*., 2020). Chloroplasts move around the cell on actin microfilaments, but

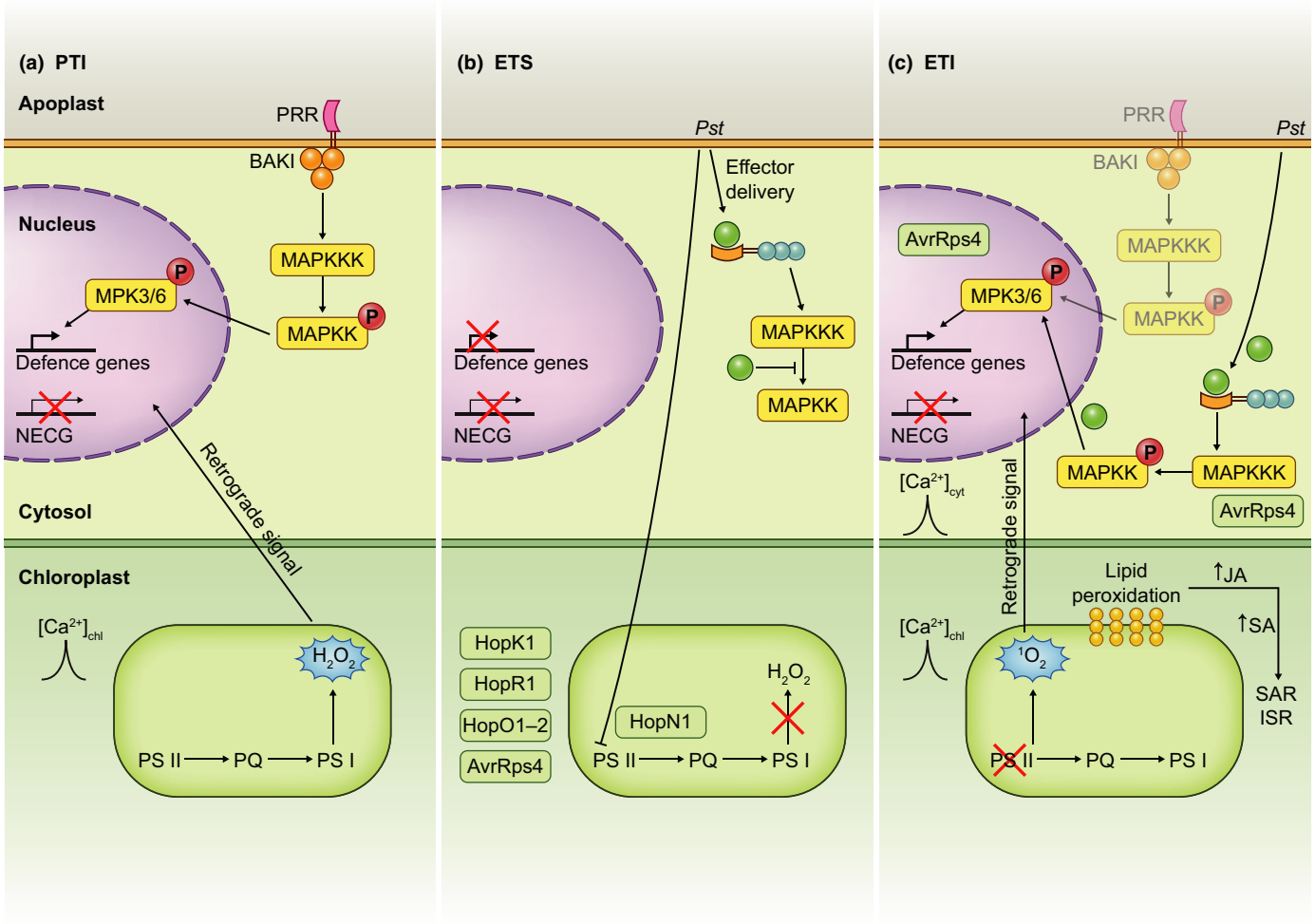

Fig. 6 Are photosystems I and II specifically targeted in disease and defence? (a) Rapid PAMP triggered immunity (PTI) responses upon PAMP recognition activate MAPK signalling and transcription, with strong suppression of *NECGs* (*Nuclear Encoded Chloroplast Genes*) relative to mock treatment (*c.* 30–40% of all differentially expressed genes) within 2 h of challenge. Early chloroplast responses include calcium spiking induced by CAS and increased H_2O_2 generation at photosystem I (PSI) which may act as a retrograde signal. (b) Effector-triggered suppression of PTI (ETS) can occur by interfering with the pattern recognition receptor, at the coreceptor complex, through modulation of MAPK signalling, via transcriptional reprogramming or directly within the chloroplast, but probably a combination thereof. Early ETS responses include attenuation of MAPK signalling and reprogramming of *NECG* expression. In the chloroplast, calcium spiking and reactive oxygen species (ROS) generation are suppressed by either interference with photosystems themselves or electron transfer between PSII and PSI. Most direct effectors targets remain unidentified but virulence strategies of diverse pathogens include direct or indirect targeting of Thylakoid formation 1 (see Fig. 3). (c) During effector triggered immunity (ETI) many of these virulence processes are overridden, with effector recognition inducing a stronger and sustained activation of MAPK signalling, an increase in $[Ca^{2+}]_{cyt}$ and rapid collapse of the quantum efficiency of PSII (F_v/F_m), which is associated with an increase in $^1O_2$ generation at PSII. This results in lipid peroxidation and appears to be the catalyst for generation of local and systemic signalling molecules. Hop, Hrp-dependent *outer* protein; NECG, nuclear encoded chloroplast genes; PRR, pattern recognition receptor; BAK1, BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 – an exemplar PRR coreceptor: CAS, calcium sensing protein; PQ, plastocyanin. SAR, systemic acquired resistance; ISR, induced systemic resistance.

there is compelling evidence which shows that stromule formation (finger-like tubular stroma-filled chloroplast extensions) is mainly microtubule-dependent (Caplan *et al.*, 2015; Erickson *et al.*, 2018). However, treatment with the microtubule-depolymerizing agent oryzalin indicates additional microtubule-independent stromule formation with each type characterized also by its speed of movement (Erickson *et al.*, 2018).

1. Stromules and perinuclear chloroplast movement – ROS as a retrograde immune signal?

Systematic studies of chloroplasts during pathogen challenge are limited. Pioneering work on the TMV N resistance protein/TMV p50 effector demonstrated a cytosolic interaction of N with chloroplastic localized N Receptor Interacting Protein 1 (NRIP1) (Caplan *et al.*, 2008). N-mediated ROS-induced stromules in a CHloroplast Unusual Positioning 1 (CHUP1)-dependent manner (Fig. 5a) (Caplan *et al.*, 2015). While stromules can be induced *in vitro*, indicating this is a chloroplast autonomous response, actin microfilament remodelling to facilitate perinuclear chloroplast movement appears to be an active ETI strategy to establish a conduit for possible retrograde ROS (or metabolite) signals (Fig. 5a) (Caplan *et al.*, 2015; Kumar *et al.*, 2018; Park *et al.*, 2018a; Park *et al.*, 2018b; Fernandez & Burch-Smith, 2019).

Stromules were observed following flg22 treatment, but not 20 hpi with *PsthrcC* (Caplan *et al.*, 2015). This apparent anomaly

may represent a timing issue as cROS is produced during early PTI (4–5 hpi with *PsthrpA*; de Torres Zabala *et al.*, 2015 (Fig. 5b)). Strikingly, PTI-induced cROS, as determined by H_2DCFDA staining, was only detected in perinuclear chloroplasts or those with stromules that appear to physically contact the nucleus following *PsthrpA* challenge. Interestingly, chloroplasts staining for ROS were significantly smaller than the others (Fig. 5b), suggesting heterogeneity in chloroplasts as reported for high light responses (Exposito-Rodriguez *et al.*, 2017), possibly a direct consequence of stromule formation. Additionally, there is evidence for chloroplast aggregation late in successful infections, as illustrated in Fig. 5(c) and described by Hutt *et al.* (2017). As many of these studies use different cell types (epidermal vs mesophyll), the importance of cell type on chloroplast function and chloroplast heterogeneity in specific pathogen immune responses requires further investigation. Chloroplasts also appear to be recruited to the EHM in *P. infestans* infections of *N. benthamiana*, where the anchoring of chloroplasts to the EHM is also CHUP1-mediated (Toufexi *et al.*, 2019). Silencing of *CHUP1* reduced chloroplast recruitment to the EHM, reduced stromule formation and led to higher levels of *P. infestans* hyphal growth, reinforcing the importance of CHUP1 and highlighting a role for chloroplast dynamics in establishment of plant immunity.

Thus, organization of chloroplasts during infection is typified by perinuclear chloroplast localization and the CHUP1-dependent extension of stromules toward the nucleus, each of which provide a physical basis for retrograde signalling (Erickson *et al.*, 2018; Mullineaux *et al.*, 2020). Indeed, perinuclear positioning of chloroplasts in immunity appears generic, being reported during viral infections (Fig. 5a) and in both avirulent (*Pst*), virulent (*PsthopQ1-1*) and *Agrobacterium tumefaciens* challenges of *N. benthamiana*, transient expression of effectors or viral proteins such as p50, or following exogeneous application of ROS (Erickson *et al.*, 2014; Caplan *et al.*, 2015; Ding *et al.*, 2019). Pathogen effects on stromule formation and chloroplast–nuclear association is remarkably similar to cROS-mediated high light responses (Exposito-Rodriguez *et al.*, 2017) and oxidative stress imposed by silencing of *NTRC* (Brunkard *et al.*, 2015).

Recent evidence for effector suppression of stromules comes from studies with the *Xcv* E3 ubiquitin ligase effector XopL. Overexpression of XopL but not an XopL E3 ubiquitin ligase mutant in *N. benthemiana* abolished stromule formation in lower epidermal cells induced by *A. tumefaciens* (Erickson *et al.*, 2014; Erickson *et al.*, 2018). By contrast, XopQ, known to elicit ETI in *N. benthemiana*, increased stromule formation by over 50%. Notably, perinuclear chloroplast localization was still observed with XopL overexpression, implying nuclear recruitment of chloroplasts and formation of stromules to be independent mechanisms in immunity.

VIII. Functional significance of suppression nuclear-encoded chloroplast genes *(NECGs)*

While suppression of *NECGs* has been reported previously (e.g. Bilgin *et al.*, 2010), a detailed time course comparing *Pst* with its type III secretion-deficient *hrpA* mutant revealed that wholesale suppression of *NECGs* was a PTI response, with *c.* 35% of all differentially suppressed genes within 3 hpi representing *NECGs* (de Torres Zabala *et al.*, 2015; Lewis *et al.*, 2015). This appears to indicate an active defence response to prioritize defence at the expense of growth. Notably, neither *hrpA* (nor flg22) challenge markedly affected Chl fluorescence parameters (de Torres Zabala *et al.*, 2015), yet within 3 hpi, *Pst* effectors differentially regulate a subset of *hrpA*-suppressed *NECGs* (Fig. 6). These transcriptional changes occur in parallel to suppression of cROS and before measurable differences in F_v/F_m or decrease in photosynthesis rate (de Torres Zabala *et al.*, 2015).

A meta-analysis of rice transcriptomic datasets also reported extensive downregulation of *NECGs* under both biotic and abiotic stress (Cohen & Leach, 2019). Considering the 11 diverse datasets and disparate temporal sampling, a core set of 85 photosynthesis-related genes were identified as suppressed across eight experiments. Thus, rapid transcriptional suppression of *NECGs* is a core response to retrograde stress signals, possibly representing a universal strategy to maximize resource allocation to defence by short-term attenuation of photosynthetic capacity, but possibly collaterally decreasing the capacity to repair effector targets.

Increasing evidence suggests that MAPKs mediate the transcriptional reprogramming of *NECGs*. MAPKs are rapidly activated following PAMP recognition and the subsequent apoplastic ROS burst (Meng & Zhang, 2013) (Fig. 6a). MAPKs can be induced by ROS but can themselves modulate ROS production. A body of evidence is emerging that the MPK3/MPK6 pathway also orchestrates ETI responses downstream of R protein activation that contribute to elevated cROS. Conditional activation of tobacco MPK3/6 orthologues SIPK/Ntf4/WIPK led to rapid, light-dependent suppression of CO_2 fixation, resulting in excess excitation energy, the generation of cROS and HR-like cell death (Liu *et al.*, 2007). ETI induced by constitutively active *Nicotiana tabacum* MAPK kinase 2 ($NtMEK2_{DD}$) led to sustained activation of MPK3/MPK6 in *Arabidopsis* (Fig. 6c). Similarly, conditional induction of *AvrRpt2* activated MPK3/MPK6, resulting in a rapid inhibition of PSII and accumulation of singlet oxygen and H_2O_2 in chloroplasts (Su *et al.*, 2018). How MAPKs impose specificity in modulating chloroplast immunity and transcriptional regulation of *NECGs* requires further investigation. It has recently been shown that fluctuating light activates local and systemic transcriptional reprogramming, including overrepresentation of genes involved in photoprotection, photosynthesis and photorespiration (Kumar *et al.*, 2018; Schneider *et al.*, 2019). Whether MPK6 integrates the retrograde signals to drive this adaptive, photoprotective response remains to be determined.

1. Emerging examples of indirect transcriptional modulation of ETI- and PTI-mediated chloroplast immunity

Here we review two examples of chloroplast immunity impacted by differential gene regulation. The first involves ETI activation of the *P. infestans* R protein Rpi-vnt1.1 by its effector AVRvnt1, which depends on light-driven alternative promoter selection. Light is required for expression of full-length tomato and potato *glycerate 3-kinase* (*GLYK*) transcripts encoding a chloroplast transit sequence.

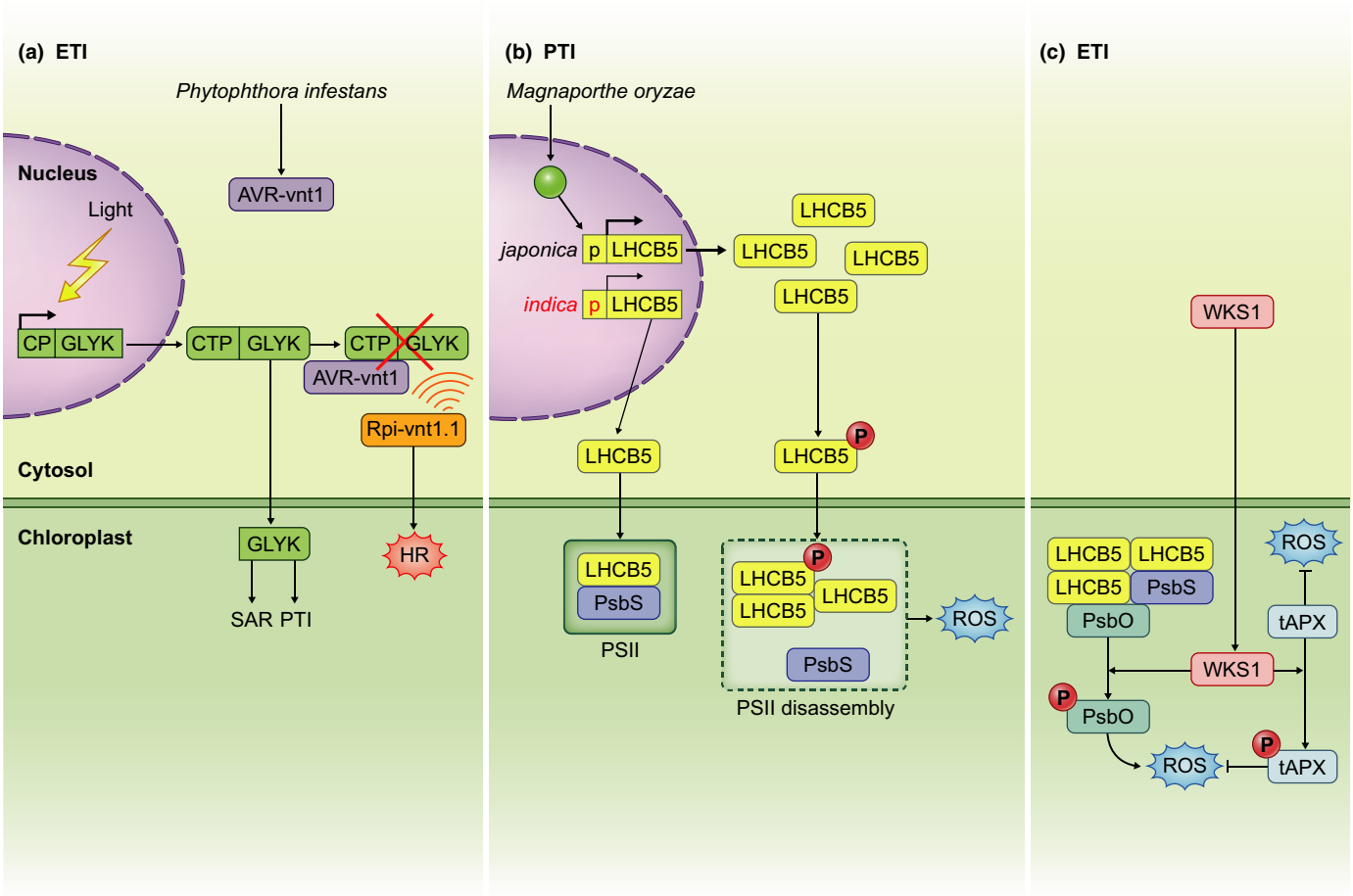

Fig. 7 Differential regulation of nuclear encoded chloroplast genes impact immunity. (a) A chloroplast targeting sequence, encoded by light-driven alternative promoter selection of the solanaceous glycerate 3-kinase (GLYK) transcript, provides a cytosolic target for the *Phytophthora infestans* effector AVRvnt1, which is recognized by the Resistance to *P. infestans* protein Rpi-vnt1.1. AVRvnt1 binds to GLYK's chloroplast targeting sequence (CTP), intercepting its trafficking to chloroplast where it plays a role in basal and systemic immunity. Depletion of GLYK is indirectly sensed by Rpi-vnt1.1 activating effector triggered immunity (ETI). (b) Infection by the rice blast fungus *Magnaporthe oryzae* activates PAMP triggered immunity (PTI) in the rice variety *japonica* (but not *indica*). This is due to a light-dependent expression polymorphism in the promoter of *Light Harvesting Complex of Photosystem II 5* (*LHCB5*). LCHB5 is cytoplasmically phosphorylated at Thr24 of its chloroplast transit sequence, leading to accumulation of both LHCB5 and superoxide in the chloroplast and enhanced basal immunity. Phosphorylated LHCB5 is predicted to form a trimeric complex, disrupting its binding to PsbS, which leads to reduced electron transfer, increased cROS and enhanced basal resistance. (c) WKS1 (Wheat Kinase START 1), an atypical resistance protein encoded at the YR36 (Yellow Rust resistance) locus, confers partial race-nonspecific resistance to *P. striiformis* f. sp. *tritici*, is N-terminally processed and localizes to the chloroplast where it phosphorylates the thylakoid-associated ascorbate peroxidase (tAPX). WKS1 also binds and phosphorylates the PSII component PsbO, decreasing its ability to bind to the supercomplex and resulting in a gradual accumulation of reactive oxygen species (ROS). SAR, systemic acquired resistance; HR, hypersensitive response.

AVRvnt1 binds to this chloroplast-targeting sequence and activates resistance (independent of GLYK kinase activity), impairing accumulation of GLYK in both total and chloroplast fractions of potato (Gao *et al.*, 2020). This is somewhat analogous to the TMV N–NRIP1 interaction described above (Caplan *et al.*, 2008), but in this case AVRvnt1 intercepts GLYK's trafficking to the chloroplast, the depletion of which (probably via proteasomal degradation) is indirectly sensed by Rpi-vnt1.1 activating ETI (Fig. 7a).

The second example requires both differential expression of the rice *Light Harvesting Complex of Photosystem II 5* (*LHCB5*) and its light-dependent phosphorylation. During infection by the rice blast fungus *Magnaporthe oryzae*, *japonica* but not *indica* rice varieties show elevated PTI due to a simple nucleotide polymorphism in the *japonica LHCB5* promoter leading to increased expression of *LHCB5* (Liu *et al.*, 2019). Cytosolic phosphorylation of LHCB5's chloroplast transit sequence on Thr24 leads to accumulation of both LHCB5 and superoxide in the chloroplast and enhanced basal immunity. Interestingly, LHCB5 was not phosphorylated during ETI. *LHCB5* overexpression lines were more resistant, and RNAi knockdown lines were more susceptible, to *M. oryzae*. LHCB5 binds PsbS, a thylakoid sensor that is involved in nonphotochemical quenching (NPQ). As phosphorylated LHCB5 accumulating in the chloroplast can form a trimeric complex, the authors predicted that during *M. oryzae japonica* infection PsbS binding is disrupted, resulting in decreased electron transfer, increased cROS and enhanced basal resistance (Fig. 7b) (Liu *et al.*, 2019). This is supported by studies on *Arabidopsis* and rice plants deficient in PsbS, which have higher levels of cROS with rice mutants showing enhanced resistant to *M. oryzae* (Zulfugarov *et al.*, 2014). This may help mechanistically explain the results of

Gohre *et al.* (2012) where the observed flg22-induced decrease in PsbS abundance may be associated with increased cROS (Gohre *et al.*, 2012).

2. Direct targeting of 'resistance' proteins to the chloroplast

Given effector localization to the chloroplast, it is not unreasonable to propose classical R proteins to be associated with the chloroplast to monitor activity, and R proteins have been experimentally predicted to be chloroplast-associated (http://suba.live/). Indeed, the atypical chloroplast-localized Wheat Kinase START 1 (WKS1) confers partial race-nonspecific resistance to *P. striiformis* f. sp. *tritici* and is encoded at the *YR36* (*Yellow Rust resistance*) locus. N-terminally processed WKS1 localizes to the chloroplast, binding to and phosphorylating both thylakoid-associated ascorbate peroxidase (tAPX) potentially restricting cROS detoxification (Gou *et al.*, 2015) and PSII component PsbO, decreasing its ability to bind to the supercomplex (Wang *et al.*, 2019) (Fig. 7c). Both *psbo-A1* mutant and RNAi lines exhibited induction of chlorosis and reduced *P. striiformis* f. sp. *tritici* growth (Wang *et al.*, 2019). The authors concluded that WKS1 initially triggers chlorosis by phosphorylating PsbO, and the gradual accumulation of ROS (exacerbated by the phosphorylation of tAPX) induces cell death. It is currently unknown whether WKS1 is a target for *P. striiformis* f. sp. *tritici* effectors. Thus, the PSII supercomplex is emerging as a common effector target, as further evidenced by HopN1 targeting of PsbQ (Rodriguez-Herva *et al.*, 2012).

IX. Concluding remarks

Chloroplasts are a central hub in plant metabolism, enabling them to act as environmental sensors and communicate *via* a diversity of retrograde signals to the nucleus. It is now clear that chloroplasts play an essential role in plant immunity, and effectors from diverse pathogens have evolved to directly or indirectly target chloroplast function. At the biochemical level, the underlying mechanisms are complex, involving chloroplast-sourced oxylipins, hormones, hydrogen peroxide and singlet oxygen. An emerging theme is that PTI is associated with simultaneous repression of *NECG*s and induction of cROS, predominantly generated at PSI. Effector-mediated suppression includes modulating *NECG*s, and manipulating hormonal balance and various strategies to attenuate cROS via disassembly of the photosystems, although a detailed understanding of this remains elusive. Recent evidence suggests that plant resistance proteins can monitor perturbations to chloroplast homeostasis or recognize chloroplast-targeted effectors to activate ETI. Although further evidence is needed, it appears that in contrast to PTI, ETI drives $^1O_2$ generation via PSII disassembly, the resultant lipid oxidation products contributing to HR. Impairment of photosystem function is potentiated by chloroplast-targeted effectors, some of which have been shown to interact with components of the photosystems likely to affect their function and stability. Furthermore, pathogen infection elicits chloroplast repositioning and formation of stromules that might facilitate retrograde signalling. Not considered in this review, but equally important, are the interacting roles of NO and interorganellular interactions with mitochondria (which have well-known roles in cell death) and peroxisomes.

Further challenges in this relatively embryonic field are multiple. We need to better understand the role of the multiple retrograde chloroplast to nucleus signalling pathways, in addition to ROS, which have been proposed to influence light response, and how these might interact with pathogens (Vogel *et al.*, 2014). Furthermore, in some cases ROS production could be a side reaction associated with other changes that comprise the actual signalling mechanism. This would not be easy to resolve but should be considered when assessing results. The challenge of understanding the relationship between the production of ROS by organelles and from the initial apoplastic PAMP-induced oxidative burst will require the use of probes with high spatial and chemical specificity. It will also require understanding the chloroplast targets manipulated by pathogens to suppress immunity.

Identifying the chloroplast targets of effectors and characterizing their interaction will not only provide important insight into how pathogens have evolved to target chloroplast immunity, but may potentially identify new herbicide leads. Aside from this, other particularly fundamental questions remain. Does the increasingly observed heterogeneity in size and positioning of chloroplasts in the cell reflect different metabolism and signalling roles in response to pathogens? How are PTI-induced cROS generated and how many chloroplasts need to respond to confer effective immunity? How many effectors, both in number and in diversity, need to target a (specific) chloroplast to suppress cROS? That being the case, do R proteins effectively guard chloroplasts?

Answers to these and other questions will not only contribute to fundamental understanding of chloroplast biology, but place the chloroplast at the forefront of endeavours to develop crops with improved pathogen resistance.

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